Assessing brain-derived neurotrophic factor as a novel clinical marker of endometriosis

Jocelyn M. Wessels, Ph.D.,[a] Vanessa R. Kay, B.Sc.,[a] Nicholas A. Leyland, M.D.,[a] Sanjay K. Agarwal, M.D.,[b] and Warren G. Foster, Ph.D.[a]

[a] Department of Obstetrics and Gynecology, McMaster University, Hamilton, Ontario, Canada; and [b] Department of Reproductive Medicine, University of California, San Diego, La Jolla, California

Objective: To evaluate novel clinical markers of endometriosis including the neurotrophins brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin 4/5 (NT4/5) and compare them to others previously reported in the literature including cancer antigen 125 (CA-125) and C-reactive protein (CRP).
Design: Prospective study.
Setting: University hospital.
Patient(s): One hundred thirty-eight women were prospectively and consecutively recruited (April 2011–April 2015; cases: undergoing endometriosis surgery, n = 96; controls: benign gynecological surgery, n = 24 combined with healthy women, no history of pelvic pain, not undergoing surgery, n = 18).
Intervention(s): Collection of peripheral blood, gynecological and demographic information, eutopic biopsy in women undergoing laparoscopy.
Main Outcome Measure(s): Circulating BDNF, NGF, NT4/5, CA-125, and CRP were quantified by ELISA.
Result(s): Plasma concentrations of BDNF were significantly greater in women with endometriosis (1,091.9 pg/mL [640.4–1,683.1]; n = 68, untreated) than in controls (731.4 pg/mL [352.1–1,176.2]; n = 36), whereas circulating NGF, NT4/5, CA-125, and CRP were not different. When assessed for their ability to differentiate between women with revised Classification of the American Society of Reproductive Medicine stage 1 and 2 or stage 3 and 4 disease and controls, BDNF was the only putative marker able to identify stage 1 and 2 disease, with a sensitivity and specificity of 91.7% and 69.4%, respectively, using an arbitrary cutoff value of 1,000 pg/mL. We also demonstrated that circulating BDNF in women with endometriosis who were receiving ovarian suppression for disease was equivalent to that in the control group. This suggests that BDNF may also offer the opportunity to monitor patient response to treatment.
Conclusion(s): Plasma BDNF is a potentially useful clinical marker of endometriosis that is superior to NGF, NT4/5, CA-125, and CRP. (Fertil Steril® 2016;105:119–28. ©2016 The Authors. Published by Elsevier Inc. on behalf of the American Society for Reproductive Medicine. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Key Words: Biomarker, CA-125, C-reactive protein, endometriosis, neurotrophin

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Endometriosis is a chronic gynecological disease of unknown etiology characterized by the presence of endometrial fragments at ectopic locations (1, 2). It affects approximately 10% of women of reproductive age from all ethnicities and is a major cause of severe pelvic pain, suffering, infertility, and hysterectomy (2–5). In the absence of a suitable diagnostic marker, the interval between onset of symptoms of endometriosis and confirmed diagnosis by laparoscopy is 11.7 years in the United States (6). Lost time from work, costly medical interventions, and surgical procedures all contribute to endometriosis being one of the largest health care expenditures, with the annual cost of treatment and patient care reaching approximately $22 billion in the United States (7–9) and

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Reprint requests: Warren G. Foster, Ph.D., Department of Obstetrics and Gynecology, HSC-3N52D McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada (E-mail: fosterw@mcmaster.ca).

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$1.8 billion in Canada (10). Significantly more resources are spent on endometriosis than on other chronic conditions (migraines, asthma, and Crohn's disease) (8), and thus identification of a clinical marker of disease remains a top priority.

Emerging evidence suggests an important role for the neurotrophins, a family of growth factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3, (NT-3), and neurotrophin 4/5 (NT4/5), in uterine physiology (11, 12) and endometrial pathology (13–17). Results of a small study suggest that women with endometriosis have elevated circulating BDNF concentrations compared with healthy controls, which decreased after surgical removal of lesions (18). However, this study was limited to patients with stage 1 and 2 disease only, and controls were healthy women alone. Moreover, the prior study was limited to BDNF, and thus it is unknown how well BDNF would compare to other clinical markers in this population. Subsequently, protein expression for BDNF and its high affinity receptor were found to be greater in the uterus of women with endometriosis compared with in disease-free controls (13, 16). Therefore, the objectives of this prospective case-control study were to assess the suitability of circulating concentrations of neurotrophins including BDNF, NGF, and NT4/5 as independent clinical markers of endometriosis and to contrast our results with other putative clinical markers of endometriosis including cancer antigen 125 (CA-125) and C-reactive protein (CRP) in the same population of women. Herein we present the results of our interim analysis of the study data.

MATERIALS AND METHODS

Study Participants

One hundred thirty-eight women were recruited and screened for inclusion in the study (Supplemental Fig. 1). One hundred twenty women undergoing gynecological laparoscopy between April 2011 and April 2015 for pelvic pain thought to be due to endometriosis were prospectively and consecutively recruited. Of these, 96 were found to have endometriosis (cases, n = 96) and 24 were diagnosed with other benign gynecological conditions (symptomatic controls, n = 24). Eighteen women with no history of pelvic pain and not undergoing surgery were also recruited (asymptomatic controls, n = 18). The study exclusion criteria were individuals unable to provide consent, age under 18, pregnancy, or a diagnosis of adenomyosis in the control group (three of 138). Adenomyosis was diagnosed by the gynecological surgeon using pelvic ultrasound and surgical evidence of disease. Women receiving hormone therapies for endometriosis in the 3 months before study enrollment were excluded from the untreated group of cases but were included in the treated group of cases (Supplemental Fig. 1) to determine the effect of endometriosis treatment on circulating clinical markers. All participants completed demographics and gynecologic questionnaires from which menstrual cycle length, date of last menstruation, and pelvic pain were determined. Pelvic pain was assessed using a nonstandardized pelvic pain test consisting of four separate 5-point questions on a visual analog scale and totaled out of 20. Menstrual cycle stage was determined by uterine biopsy for women undergoing surgery and using the date of last menstruation for those not undergoing surgery. During laparoscopic surgery women were categorized as a case or symptomatic control by a gynecological surgeon, and the diagnoses were confirmed by pathology reports. The stage of endometriosis was determined by the surgeon during surgery according to the revised Classification of the American Society of Reproductive Medicine (rAFS) (19). This study was approved by the Research Ethics Board, McMaster University (Institutional Review Board no. 06-064, 14-066-T), and all participants provided written informed consent before surgery.

Peripheral blood was collected from participants into plasma and serum separator tubes (BD Canada) by a nurse at McMaster University Medical Centre. As our initial primary markers/endpoints are found in plasma, serum was not collected from most asymptomatic controls (n = 16) nor from a few other cases (n = 11). Blood was placed on ice, transferred to the laboratory, and centrifuged at 3,000 rpm, and approximately 200 μL of plasma or serum was aliquoted into 1.8 mL cryovials (Sarstedt) and frozen at −80°C.

BDNF Assay

Plasma samples were thawed at room temperature, and BDNF concentrations were quantified in triplicate using the BDNF Emax immunoassay ELISA (Promega) following the manufacturer's protocol. Briefly, 96-well NUNC maxisorp plates (Fisher Scientific) were coated with antihuman BDNF antibody overnight. Freshly thawed plasma samples were diluted 1:10 with the provided sample buffer. After incubation, the absorbance was read at 450 nm within 30 minutes using the Biotek Synergy spectrophotometer (Fisher Scientific). The kit sensitivity was 15.6 pg/mL.

NGF and NT4/5 Assays

Serum samples were thawed at room temperature, and circulating NGF was quantified in duplicate in neat serum using the human β-NGF Mini ELISA Development Kit (Peprotech) following the manufacturer's protocol. Incubations for the sample and detection antibody were lengthened to 3 and 2.5 hours, respectively. The kit has a sensitivity of 16 pg/mL. NT4/5 was quantified in duplicate using the Human NT-4 ELISA (RayBiotech), which has a sensitivity of 2 pg/mL. The plates were incubated with neat serum overnight at 4°C and according to the manufacturer's protocol. ELISAs were read as above.

CA-125 and CRP Assays

Circulating CA-125 and CRP were quantified in duplicate using the Human CA-125/MUC16 Quantikine ELISA Kit (R&D Systems) and Human CRP ELISA (Life Technologies), following the manufacturers' protocols. Plasma samples were thawed at room temperature and diluted 1:3 (CA-125) or 1:4,000 (CRP) with the diluent provided. The sensitivity of the CA-125 and CRP assays is 0.035 U/mL and 10 pg/mL, respectively. ELISAs were read as above.

Data and Statistical Analysis

Before our study, we anticipated a difference in circulating BDNF between cases and controls of approximately 250 pg/mL and an SD of plasma BDNF concentrations of 230 pg/mL, on the basis of our preliminary results and those of Giannini et al. (18). Sample size was calculated to be 15 women per group, using the two-tailed Student's *t*-test, with a power of 80% and alpha of 5%. The sample size calculation to detect differences in plasma BDNF across disease stage (control vs. stage 1 and 2 vs. stage 3 and 4) by analysis of variance was calculated to be 18 women per group, using the same parameters as above. Patient demographics were compared between cases and controls by *t*-test, Mann-Whitney rank sum test, or χ^2 (SigmaStat 3.5 Systat Software) and are presented in Table 1 as mean ± SD, median (25%–75% percentiles) or n, %. For demographics that differed significantly between cases and controls, multiple logistic regression was carried out to determine whether any of the factors were significantly associated with being classified as a case or control. Nine women were excluded from the study owing to missing samples (n = 2), nondetectable BDNF (n = 1), or a diagnosis of adenomyosis (n = 3), or they were classified as a control but taking Lupron (n = 3). After determining that there was no significant difference in circulating BDNF between asymptomatic women who were (n = 6) and were not (n = 12) on oral contraceptives (Supplemental Fig. 2A; P=.174), we combined them to increase the sample size of the control group. Symptomatic controls who were (n = 2) and were not (n = 16) on oral contraceptives were also combined (Supplemental Fig. 2B; P=.663).

Next, the concentrations of BDNF, CA-125, and CRP were compared between the symptomatic and asymptomatic control groups (Supplemental Fig. 2C, 2D, 2E, respectively) by *t*-test or Mann-Whitney rank sum test and did not differ significantly (P=.159, .950, .137, respectively). Therefore, the two control groups (symptomatic and asymptomatic) were combined into one control group for all subsequent analyses. Circulating BDNF, NGF, NT4/5, CA-125, and CRP concentrations were compared by Mann-Whitney rank sum test (cases [all stages] vs. controls), or Kruskal-Wallis one-way analysis of variance on ranks (across stage of disease and by treatment) using SigmaStat (Systat Software Inc.) and are presented in the text as medians (25%–75% percentile). Receiver operating characteristic (ROC) curves were compiled for circulating BDNF, NGF, NT4/5, CA-125, and CRP using the ROC macro in SigmaStat. P<.05 was considered statistically significant.

RESULTS

Patient Characteristics

Of the women recruited to participate in this study (n = 138), 120 underwent laparoscopic surgery from which 96 cases of endometriosis and 24 symptomatic controls (women experiencing pain due to other indications including pelvic pain no diagnostic abnormality [n = 3], benign cysts [n = 4], uterine fibroids [n = 5], adenomyosis [excluded, n = 3], chronic inflammation [n = 3], polycystic ovary syndrome [n = 3], endometrial polyps [n = 1], or epidermoid cyst [n = 2]) were identified. Three women in the control group were receiving Lupron and were thus excluded from the study (diagnoses: polycystic ovary syndrome [n = 1], fibroids [n = 1], chronic inflammation [n = 1]). An additional group of women with no history of pelvic pain (asymptomatic) not undergoing surgery were recruited as healthy controls (n = 18). After the exclusion of women with adenomyosis (n = 3), controls on Lupron (n = 3), the removal of incomplete samples (n = 2), nondetects (n = 1), and amalgamation of control groups, the final study population was 129 women: 93 cases and 36 controls (Supplemental Fig. 1).

The average age of cases was significantly higher (P=.001) than that of controls (34.7 ± 7.0 vs. 29.9 ± 8.5, respectively; Table 1), and ethnicity (P=.004), occupational status (P=.017), and smoking status (P=.031) differed between cases and controls. Self-reported pelvic pain was significantly higher in cases than in controls (three of 20 vs.

TABLE 1

Patient characteristics of women with and without endometriosis.

Characteristic	Control (n = 36)	Case (n = 93)	*P* value
Age (y), mean ± SD	29.9 ± 8.5	34.7 ± 7.0	.001
Ethnicity, n (%)			
Caucasian	28 (78)	68 (73)	.004
Asian	7 (19)	4 (4)	
Black	0 (0)	5 (5)	
Unknown	1 (3)	16 (17)	
Occupational status, n (%)			
Employed	16 (44)	53 (57)	.017
Unemployed	1 (3)	1 (1)	
Other	15 (42)	12 (13)	
Unknown	4 (11)	27 (29)	
Smoking status, n (%)			
Nonsmoker	34 (94)	70 (75)	.031
Smoker, <20 cigarettes/d	2 (6)	10 (11)	
Unknown	0 (0)	13 (14)	
Median age at first menstruation, y (25%–75%)	12 (12–13)	12 (11–13)	.639
Median duration of bleeding, d (25%–75%)	6 (5–7)	6 (4–7)	.817
Menstrual cycle stage, n (%)			
Menstrual	5 (14)	13 (14)	
Proliferative	9 (25)	19 (20)	.348
Secretory	12 (33)	20 (22)	
Unknown	2 (6)	16 (17)	
Ovarian suppression	8 (22)	25 (27)	
Median pelvic pain (self-report, 0–20) (25%–75%)	3 (2–8)	9 (6–11)	<.001
Current medical therapies, n (%)			
Hormonal contraceptives	8 (22)	9 (10)	.176
Lupron	0 (0)	16 (17)	
Nonsteroidal anti-inflammatory drug	2 (5)	15 (16)	
Narcotic analgesic	1 (3)	6 (6)	
None/other	25 (70)	47 (51)	
Stage of endometriosis, n (%)			
Minimal, 1	0 (0)	10 (11)	NA
Mild, 2	0 (0)	9 (10)	
Moderate, 3	0 (0)	10 (11)	
Severe, 4	0 (0)	64 (68)	

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nine of 20; $P \leq .001$). Multiple logistic regression analysis was conducted using the designation of "case" or "control" as the dependent variable and age, ethnicity, occupational status, smoking status, and pain as independent variables to determine their effect on the dependent variable. In this model, only pain ($P < .001$) remained significantly associated with being a case or control, while age ($P = .055$), ethnicity ($P = .265$), occupational status ($P = .461$), and smoking status ($P = .879$) were not.

Menstrual cycle stage, current medical therapies, age at first menstruation (12 [11–13] years for cases vs. 12 [12–13] years for controls; $P = .639$), and duration of bleeding in days (6 [4–7] for cases vs. 6 [5–7] for controls: $P = .817$) were not different between groups. Of the 93 cases, 68 had not received any hormone treatment in the 3 months before surgery (21 were using nonsteroidal anti-inflammatory drugs [NSAIDs] or narcotic analgesics to manage pain), and 25 were being treated for endometriosis (hormonal contraceptives [nine of 25] and Lupron [16 of 25]).

Neurotrophins, CA-125, CRP, and Endometriosis

Our data set was analyzed separately (univariate analysis) for each putative marker, first regardless of stage of disease or menstrual cycle stage. The median circulating concentration of BDNF in the plasma was significantly greater ($P = .018$) in women with endometriosis (1,091.9 [640.4–1,683.1] pg/mL; n = 68, untreated) than in controls (731.4 [352.1–1,176.2] pg/mL; n = 36; Fig. 1A). To determine whether circulating concentrations of BDNF, NGF, NT4/5, CA-125, and CRP were affected by menstrual cycle phase, the data were reanalyzed by phase (menstrual, proliferative, secretory) in untreated cases (Supplemental Fig. 3A–3D). The effect of menstrual cycle on circulating BDNF was also assessed in untreated controls (Supplemental Fig. 3F). There was no significant effect of menstrual cycle phase on circulating BDNF ($P = .648$), NGF ($P = .169$), NT4/5 ($P = .314$), CA-125 ($P = .821$), or CRP ($P = .360$) in untreated cases and no significant effect of cycle phase on circulating BDNF in controls ($P = .460$). Thus subsequent analyses were not stratified by cycle stage. Further, as pelvic pain had been found to be significantly associated with being a case or control in our preliminary statistical analysis, the relationship between pelvic pain and each putative biomarker was determined by linear regression in untreated cases and controls. No significant association with pelvic pain was observed for BDNF ($P = .307$), NGF ($P = .687$), CA-125 ($P = .613$), or CRP ($P = .152$; Supplemental Fig. 4A, 4B, 4D, 4E, respectively). However, NT4/5 was significantly associated with pain ($P = .012$; Supplemental Fig. 4C). As the majority of markers did not have an association with pain, subsequent analyses were not stratified by pelvic pain. Finally, no association between circulating BDNF and age was observed using linear regression in cases and controls (Supplemental Fig. 4F).

Serum samples were unavailable for asymptomatic women and 11 cases. However, circulating NGF in the serum of the remaining subset of untreated cases (n = 57) was 71.1 (29.7–173.4) pg/mL and was not significantly different ($P = .418$) from a subset of controls (n = 22) who had concentrations of 77.9 pg/mL (28.5–99.2 pg/mL; Fig. 1B). In the same subset, the median circulating NT4/5 in the serum was 7.9 (3.8–20.1) pg/mL, which did not differ significantly ($P = .351$) compared to women without endometriosis who had 5.2 pg/mL (0.3–24.0 pg/mL; Fig. 1C).

In women with endometriosis (n = 68, untreated), the circulating concentration of CA-125 in the plasma was 7.8 (4.0–18.9) U/mL and was not significantly different ($P = .369$) from that of women without endometriosis (n = 36) who had concentrations of 7.0 U/mL (5.1–10.5 U/mL; Fig. 1D). In the same group of women, circulating CRP did not differ ($P = .929$) between cases (2.2 [0.6–4.6] μg/mL) and controls (3.1 [0.5–3.8] μg/mL; Fig. 1E).

Neurotrophins, CA-125, CRP, and Stage of Disease

The relationship between circulating BDNF, NGF, NT4/5, CA-125, and CRP and stage of disease in women not receiving treatment for endometriosis (Fig. 2) was determined. Women with stage 1 and 2 endometriosis had significantly elevated BDNF ($P = .028$) compared with controls (1,178.6 [1,043.8–1,433.8] pg/mL vs. 731.4 [352.1–1,176.2] pg/mL, respectively; stage 1 and 2, n = 12; controls, n = 36; Fig. 2A). No significant difference in circulating BDNF was found for women with stage 1 and 2 versus stage 3 and 4 (1,178.6 [1,043.8–1,433.8] pg/mL stage 1 and 2, n = 12; vs. 1,076 [593.7–1,433.8] pg/mL stage 3 and 4; n = 56, respectively) nor between women with stage 3 and 4 disease versus the control group (1,076 [593.7–1,433.8] pg/mL vs. 731.4 [352.1–1,176.2] pg/mL, respectively). NGF (Fig. 2B) and NT4/5 (Fig. 2C) were compared across stage of disease and did not differ significantly ($P = .619$ and .463; respectively).

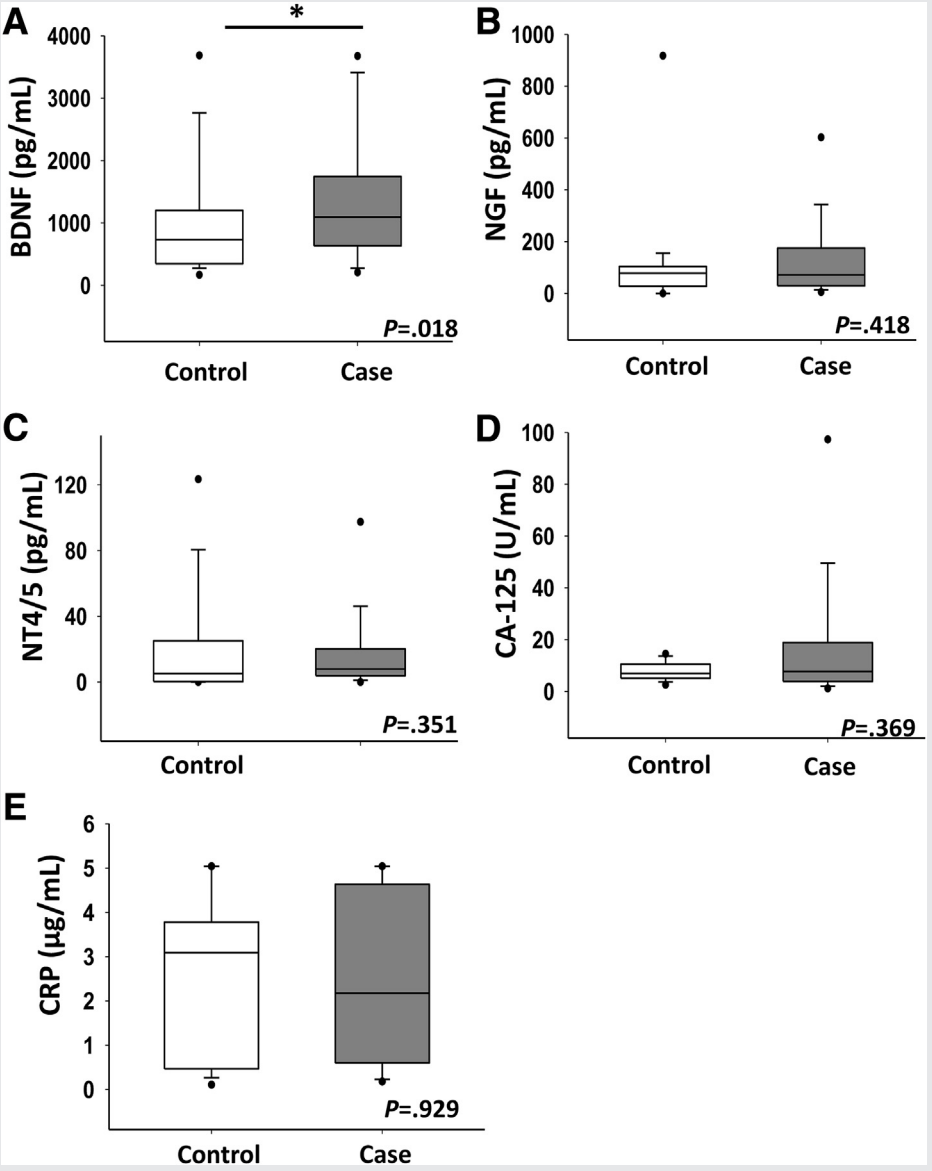
Circulating CA-125 was significantly increased in women with stage 3 and 4 endometriosis versus women with stage 1 and 2 disease ($P = .007$; 9.2 [4.8–21.7] U/mL vs. 3.7 [2.5–7.3] U/mL; n = 56 and 12, respectively; Fig. 2D). There were no significant differences between women with stage 1 and 2 or stage 3 and 4 disease and controls. Nor were significant differences in CRP observed between women with stage 1 and 2 or 3 and 4 disease and controls (3.8 [0.9–4.6], 1.8 [0.6–4.6], and 3.1 [0.5–3.8] μg/mL, respectively; $P = .638$; Fig. 2E).

ROC curves for BDNF, NGF, NT4/5, CA-125, and CRP were generated including women with stage 1 and 2 disease (n = 12) who were not receiving endometriosis treatment compared with controls (Fig. 2F). BDNF had the greatest area under the curve (0.75; $P = .009$) compared with NGF (0.54; $P = .76$), NT4/5 (0.49; $P = 1.04$), CA-125 (0.27; $P = 1.98$), and CRP (0.59; $P = .34$). Using an arbitrary cutoff value of 1,000 pg/mL, the sensitivity and specificity of BDNF as a biomarker of stage 1 and 2 disease were 91.7% (confidence interval [CI], 61.5%–99.8%) and 69.4% (CI, 51.9%–83.7%), respectively.

Neurotrophins, CA-125, CRP, and Endometriosis Treatment

The effect of treatment on circulating levels of putative endometriosis biomarkers was assessed (Fig. 3). The treated group of women had stage 1 and 2 (seven of 25) and stage 3 and 4

FIGURE 1



Putative biomarkers of endometriosis. The circulating concentration of BDNF in the plasma was significantly elevated (P=.018) in women with all stages of endometriosis who were not receiving hormone treatment or Lupron (n = 68) compared with women without endometriosis (n = 36) (**A**). Neither circulating NGF (**B**) nor NT4/5 (**C**) differed significantly between a subgroup of cases (n = 57) and controls (n = 22). Circulating CA-125 (**D**) and CRP (**E**) were quantified in the same women as BDNF. Neither CA-125 nor CRP differed between cases and controls. Statistically significant differences are denoted by an asterisk (*) above the graph. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles, respectively.

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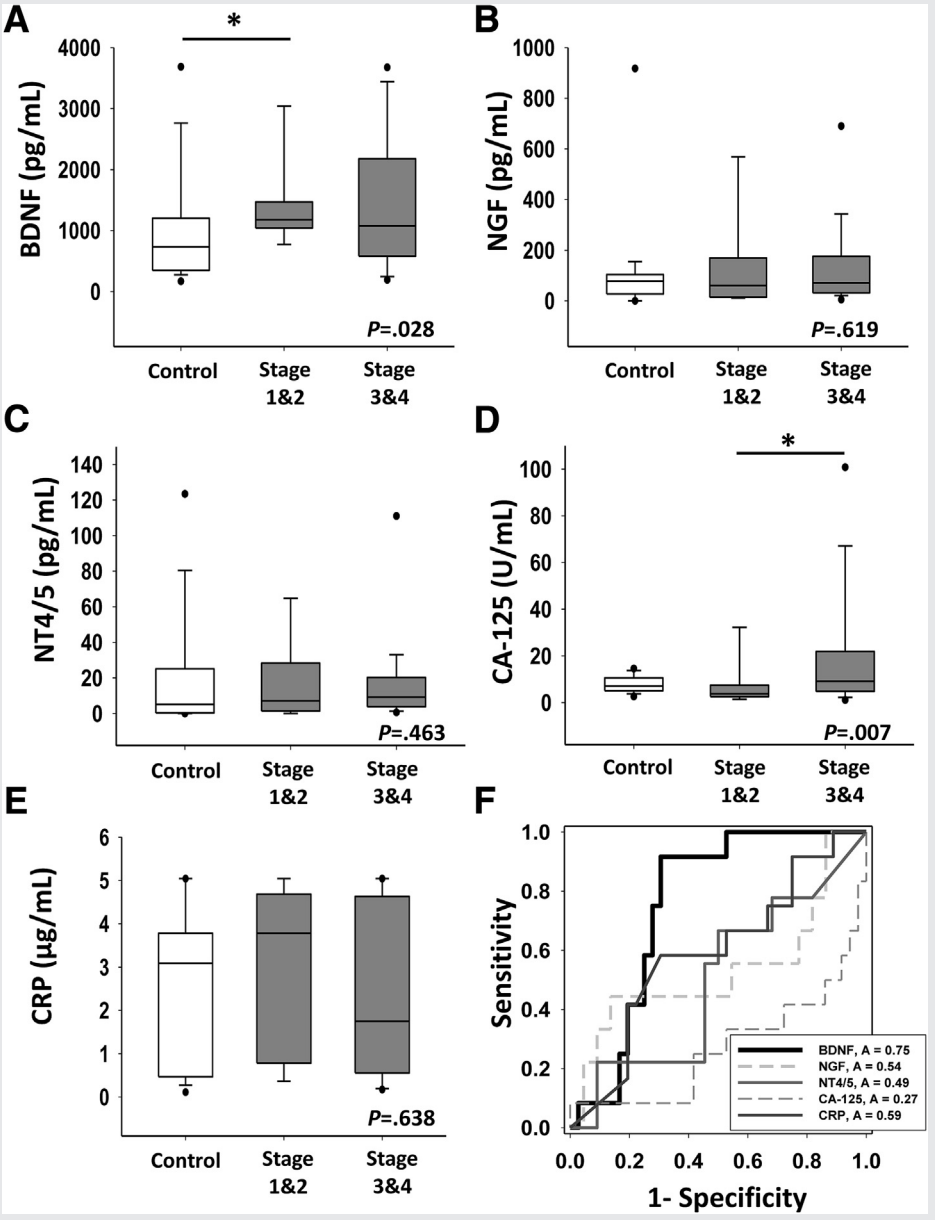
(18 of 25) disease, and treatments included oral contraceptives (nine of 25) and Lupron (16 of 25). No significant difference (P=.203) in the concentration of BDNF was observed between women on oral contraceptives and those on Lupron (Supplemental Fig. 5), thus they were grouped together and called the "treated" group in all subsequent analyses. Women in the untreated group (n = 68) were not receiving endometriosis treatment (47 of 68) or were only using NSAIDs (15 of 68) or narcotic analgesics (six of 68) to manage pain. The untreated group consisted of women in stage 1 and 2 (12 of 68) and stage 3 and 4 (56 of 68). Of the five putative markers quantified, only BDNF (Fig. 3A) demonstrated a significant difference between untreated and treated women with endometriosis and controls (1,091.9 [640.4–1,683.1] vs. 729.1 [439.7–1,488.2] vs. 731.4 [352.1–1,176.2] pg/mL, respectively; P=.025). No significant difference in circulating BDNF was observed between women treated for endometriosis and controls (P=.971). There was no effect of treatment

FIGURE 2



Neurotrophins, CA-125, CRP, and stage of disease. Women with stage 1 and 2 endometriosis (n = 12) who were not receiving treatment had significantly elevated BDNF (*P*=.028) as compared with controls (n = 36) (**A**). There were no significant differences between women with stage 1 and 2 versus stage 3 and 4 disease (n = 56) nor between women with stage 3 and 4 disease versus controls. No significant difference in circulating NGF (**B**) nor NT4/5 (**C**) was observed between groups in a subset (control = 22; stage 1 and 2 = 9; stage 3 and 4 = 48). Circulating CA-125 was significantly increased (*P*=.007) in women with stage 3 and 4 endometriosis as compared with those with stage 1 and 2 disease (**D**). No significant difference in CRP was seen between women with stage 1 and 2 or those with stage 3 and 4 disease and controls (**E**). ROC curves for BDNF, NGF, NT4/5, CA-125, and CRP were generated for women with stage 1 and 2 disease not receiving treatment for endometriosis (stage 1 and 2; n = 12) versus controls (n = 36) (**F**), and BDNF had the greatest area (*A*) under the curve (0.75; *P*=.009) compared with NGF (0.54; *P*=.76), NT4/5 (0.49; *P*=1.04), CA-125 (0.27; *P*=1.98), and CRP (0.59; *P*=.34). Using an arbitrary cutoff value of 1,000 pg/mL, the sensitivity and specificity of BDNF as a biomarker of stage 1 and 2 disease were 91.7% (CI, 61.5%–99.8%) and 69.4% (CI, 51.9%–83.7%), respectively. Statistically significant differences are denoted by an asterisk (*) above the graph. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles, respectively.

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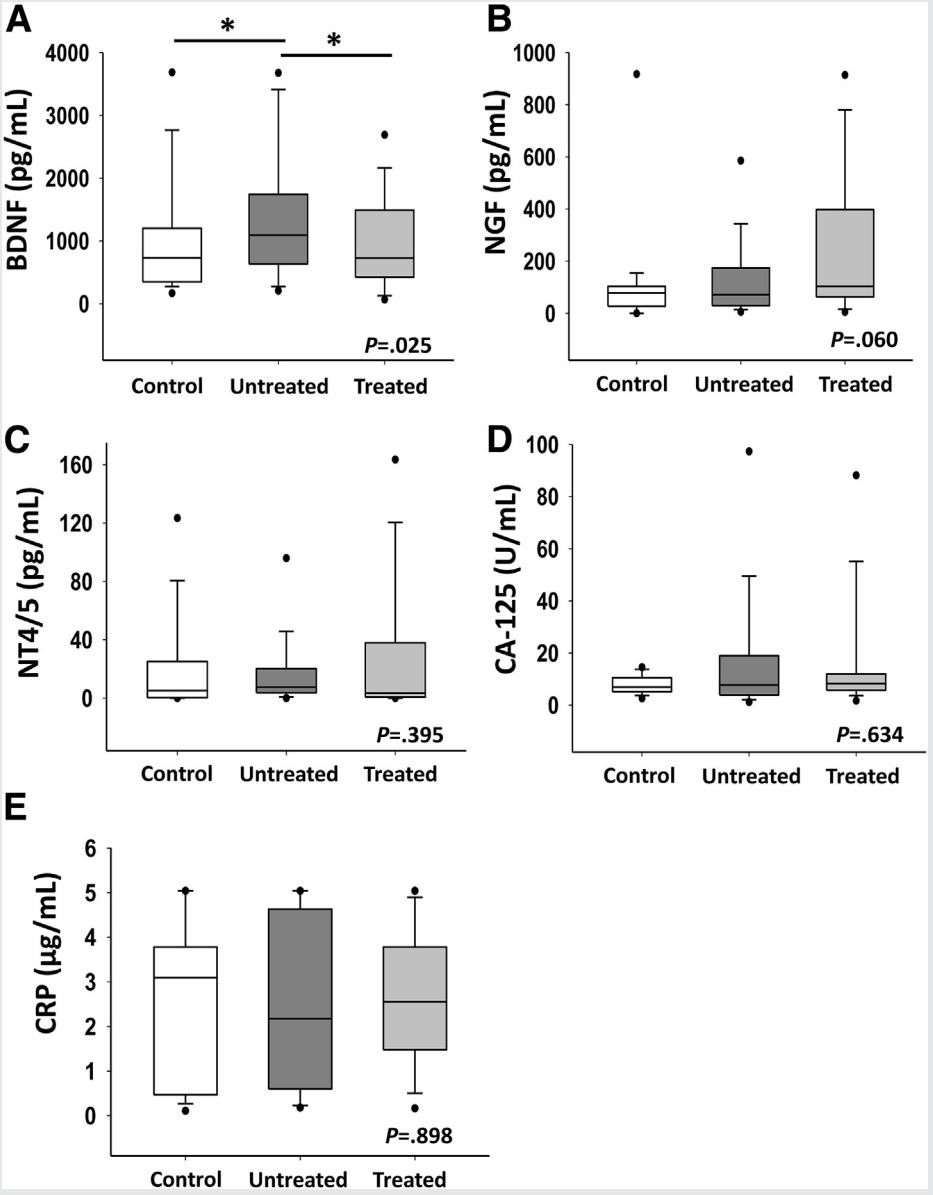
on circulating concentrations of NGF (71.1 [29.7–173.4] vs. 103.8 [70.6–346.1] vs. 77.9 [28.5–99.2] pg/mL; *P*=.060; Fig. 3B), NT4/5 (7.6 [3.8–20.0] vs. 3.5 [0.7–37.9] vs. 5.2 [0.3–24.0] pg/mL; *P*=.395; Fig. 3C), CA-125 (7.8 [4.0–18.8] vs. 8.3 [5.7–11.5] vs. 7.0 [5.1–10.5] U/mL; *P*=.634; Fig. 3D), or CRP (2.2 [0.6–4.6] vs. 2.6 [1.5–3.8] vs. 3.1 [0.5–3.8] µg/

FIGURE 3



Neurotrophins, CA-125, CRP, and endometriosis treatment. Women in the untreated group (n = 68) were not receiving endometriosis treatment, whereas those in the treated group (n = 25) were on oral contraceptives or Lupron. Circulating BDNF (**A**) was significantly elevated (*P*=.025) in women with endometriosis who were not receiving treatment compared with women receiving treatment and controls (n = 36). There was no significant difference in NGF (**B**), NT4/5 (**C**), CA-125 (**D**), or CRP (**E**) across the groups. Statistically significant differences are denoted by an asterisk (*) above the graph. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles, respectively.

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mL; *P*=.898; Fig. 3E) among untreated, treated, and control women, respectively.

DISCUSSION

The results of the present study reveal that plasma BDNF concentrations are greater in the circulation of women with endometriosis, particularly those with stage 1 and 2 disease, compared with in a control group consisting of symptomatic (women with pelvic pain but not endometriosis) and asymptomatic (healthy) women. Moreover, we demonstrated that employing BDNF as a biomarker of stage 1 and 2 disease using an arbitrary cutoff value of 1,000 pg/mL resulted in a test with high sensitivity 91.7% (CI, 61.5%–99.8%) and an acceptable specificity 69.4% (CI, 51.9%–83.7%). We also show that CA-125 is significantly elevated in women with

stage 3 and 4 endometriosis versus in women with stage 1 and 2 disease.

In this study we sought to compare BDNF to other neurotrophins including NGF and NT/4/5 and other previously studied putative markers of endometriosis CA-125 and CRP (20–22) as a single, relatively noninvasive marker of endometriosis. CA-125 was selected as a comparator because it is the most studied marker of endometriosis (20), and CRP was selected owing to its association with inflammation (20). A panel including all biomarkers combined was assessed by multiple logistic regression analysis (data not shown), however, univariate analysis of BDNF proved a better predictor of disease. The inclusion of BDNF in a panel consisting of endometriosis biomarkers other than those presented in this study is warranted and might increase the ability of a panel to detect stage 1 and 2 disease. Furthermore, our results suggest that rather than developing one panel of biomarkers to predict all stages of endometriosis, a separate panel for stages 1 and 2 and stages 3 and 4 might increase their sensitivity and specificity. Of the five putative markers described, BDNF was superior owing to its ability to detect rAFS stage 1 and 2 disease, which is often difficult to diagnose clinically, and because it was lower in women receiving ovarian suppressive therapies for endometriosis (oral contraceptives and Lupron) than in untreated women. Although not directly assessed in this study, monitoring BDNF before and during endometriosis treatment might show a relationship with treatment efficacy and provide a proxy of patient response to treatment.

Overall, we found that circulating concentrations of BDNF were significantly higher in women with endometriosis who were not receiving treatment versus in the control group. We also observed that circulating BDNF was lower in women receiving ovarian suppression to treat endometriosis as compared with untreated women. We acknowledge that it is ideal to include a 3-month hormone-free treatment period before study enrollment to eliminate the potential confounding effects of ovarian suppression. However, we suggest that the inclusion of a group of treated cases in the present study is an accurate reflection of the clinical reality. Our results are in accordance with and expand upon the findings of a prior study (18), which showed a significant elevation in plasma BDNF in women with stage 1 and 2 disease versus healthy controls and a decrease in concentration after surgical removal of lesions. However, the previous study did not explore the relationship between circulating BDNF in women with endometriosis compared with women with pelvic pain but without endometriosis (symptomatic controls), did not include women with stage 3 and 4 disease, and did not include women receiving medical therapies. Another larger study of fertility patients revealed a link between the presence of a BDNF (Met) single-nucleotide polymorphism (SNP) and increased severity of endometriosis (stages 3 and 4), which was thought to contribute to endometriosis-associated infertility (17). On the basis of our results indicating that BDNF is elevated in stage 1 and 2 disease, we hypothesize that the circulating concentration of BDNF might more accurately reflect disease activity (number of red/black lesions). This would, perhaps in part, explain the large variation in circulating BDNF in women with stage 3 and 4 disease, where adhesions and inactive lesions often predominate. Furthermore, an SNP in the BDNF gene was previously reported (17) and was associated with an increased severity of endometriosis. On the basis of our hypothesis that BDNF relates to the activity of disease, perhaps the SNP increased the number of active lesions and thus the severity of the disease. Taken together, several studies have now identified a link between BDNF and endometriosis.

We propose that an ideal clinical marker of endometriosis would be measurable in blood, sensitive and specific in identifying patients with all stages of the disease, and decrease in response to medical and surgical therapies. Our results revealed that, of all the markers studied, only plasma BDNF concentrations were higher in untreated cases than in treated cases. Although both BDNF and NT4/5 had previously been shown to be overexpressed in the eutopic endometrium of women with endometriosis versus controls (16), serum NT4/5 levels were not different between cases and controls in the present study. Thus, we propose that although neurotrophin family members are potentially important in the pathophysiology of endometriosis, only plasma BDNF shows promise as a novel clinical marker of endometriosis. Moreover, our results suggest that measurement of plasma BDNF may have value as a marker of treatment response in patients with endometriosis. We suggest that a prospective analysis of circulating BDNF in untreated women with endometriosis seeking treatment should be undertaken along with validated pain and quality-of-life questionnaires to address the utility of BDNF as a marker of patient response to treatment.

The strengths of our study include the prospective case-control design, confirmation of endometriosis diagnosis by surgery and pathology, inclusion of a treated group of women with endometriosis, and assessment of potential confounders (pain, age, menstrual cycle phase, ethnicity, occupation, and smoking status). We also consider the inclusion of a clinically relevant control group (symptomatic) as a strength of the study. Upon initial analysis, these women were not different from healthy asymptomatic controls, and thus they were merged into a single control group for subsequent analyses. Additionally, our study examined the effect of menstrual cycle phase on the putative biomarkers quantified, even though many biomarker studies do not take menstrual cycle stage into consideration during study design and/or analysis (22). In our cohort of untreated women, there was no effect of menstrual cycle phase, as determined by endometrial biopsy, on any of the putative markers quantified. Other studies have found an effect of menstrual cycle phase on endometriosis markers, in particular the elevated ratio of serum CA-125 between menses and the proliferative phase was demonstrated to be increased in women with endometriosis as compared with controls (23–25) and has been proposed as a putative biomarker. However, because CA-125 did not differ across cycle phases in our cohort, comparison of BDNF as a clinical marker against the CA-125 menses to the proliferative phase ratio could not be done. Furthermore, two studies in healthy

cycling women found a significant increase in circulating BDNF during the secretory phase (days 20–24) as compared with during the proliferative phase (days 6–8) (26, 27). However, we did not observe any difference in BDNF concentration, nor any other putative marker quantified, between phases of the menstrual cycle in our study population.

Divergent study results are likely explained by the fact that women were not recruited on specific cycle days into our study, as they had been in other studies describing an effect of menstrual cycle stage, and all samples were collected in the morning, thus avoiding diurnal variation in plasma BDNF as previously reported (24). As there was no difference between cycle phases, our data were not stratified by cycle phase. By grouping women as cases or controls and not subdividing by menstrual cycle phase we were likely biasing our results toward the null hypothesis, that there was no difference in circulating concentrations of BDNF between groups. However, we observed significantly greater BDNF concentrations in women with endometriosis than in those without, validating our decision not to stratify our data and further suggesting that the difference in concentrations might in fact be widened by using more rigorous inclusion and analysis criteria in future studies. Furthermore, the ability to quantify BDNF on any cycle day is an advantage for a clinical marker, as it can be quantified on the day a woman presents to the clinic and not delayed.

Although the results of the present study are encouraging, there are a number of important limitations. Specifically, as a tertiary care center for endometriosis, the majority of our patient population presents with advanced-stage disease and thus the sample size for stage 1 and 2 endometriosis is limited. Since there is generally little rationale to operate on women with stage 1 and 2 disease, we are restricted to incidental findings of endometriosis in women undergoing laparoscopy for other indications.

Another potential limitation is that our asymptomatic controls did not undergo surgery to rule out a diagnosis of endometriosis. However, if any of the asymptomatic controls were to have endometriosis, our results would be biased toward the null hypothesis, that no difference in circulating BDNF exists between women with and without endometriosis. Thus, we are confident in including these women in our study. Finally, the results of this study pertain to a particular study population, and thus our results need to be independently validated to add external validity.

In conclusion, plasma BDNF is superior to NGF, NT4/5, CA-125, and CRP in our cohort of women as a single, relatively noninvasive clinical marker of endometriosis. Further, BDNF has promising sensitivity 91.7% and specificity 69.4% for detecting stage 1 and 2 endometriosis and may also provide an indicator of patient response to treatment.

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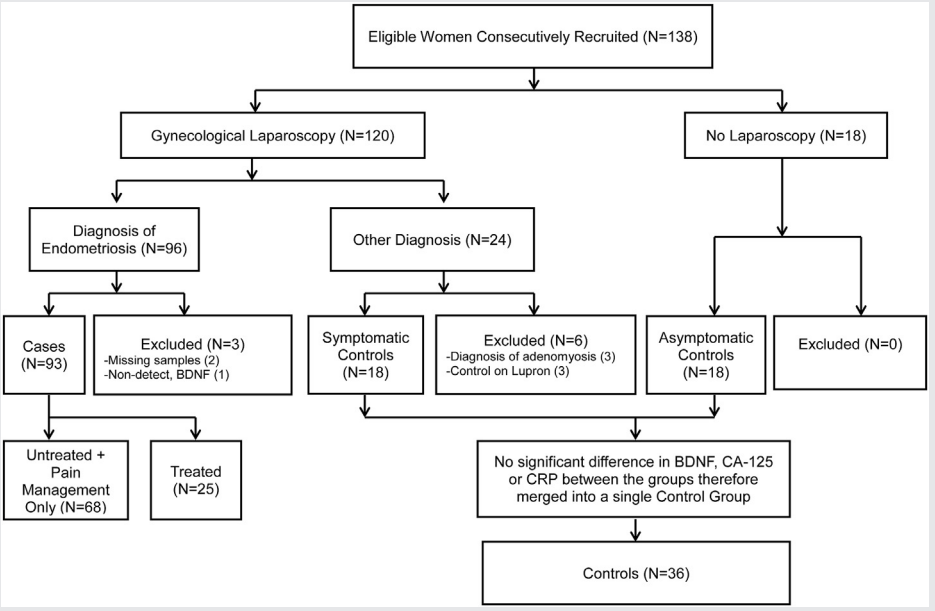
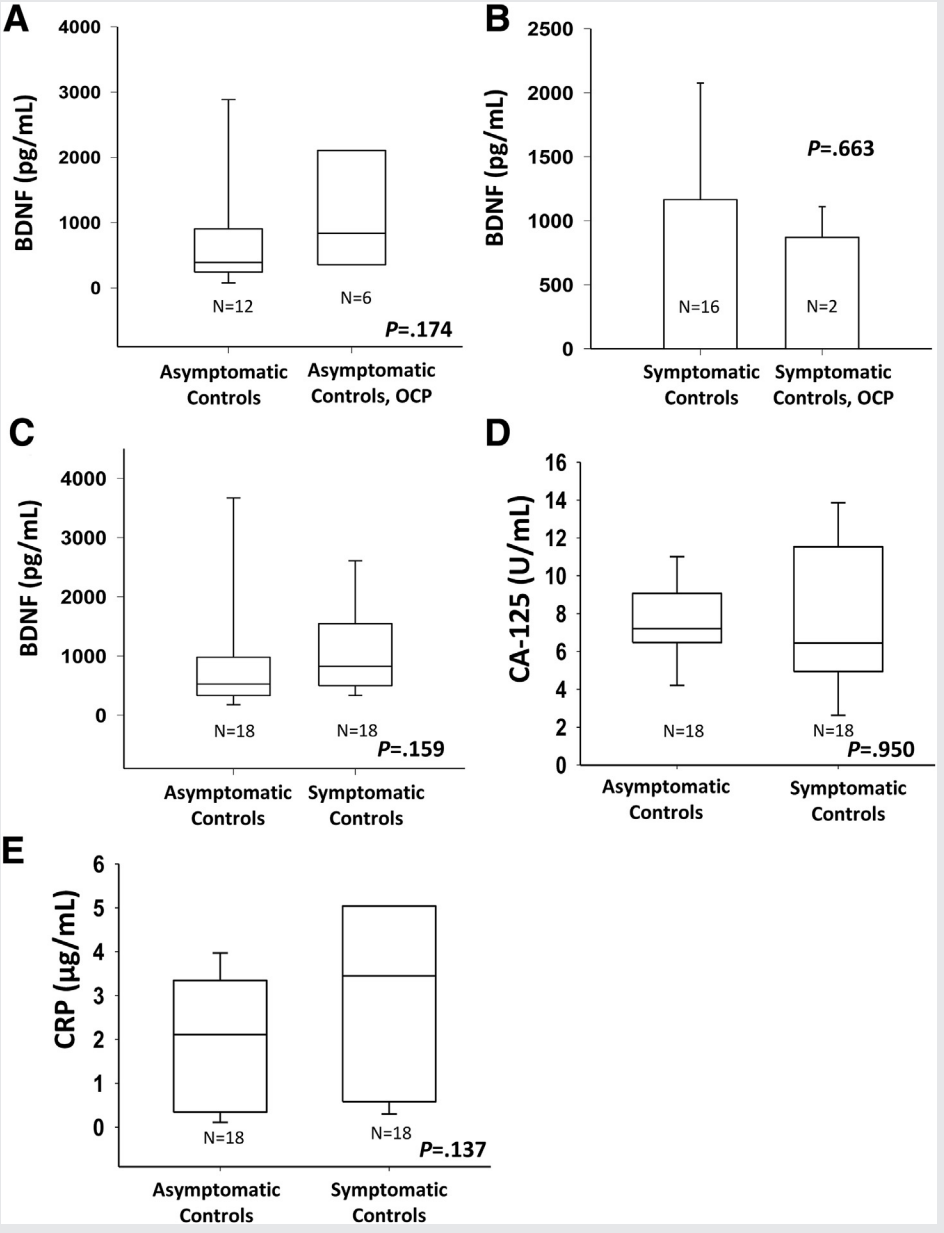
SUPPLEMENTAL FIGURE 1



Study design. One hundred thirty-eight women were prospectively and consecutively recruited to participate in the study. Gynecological laparoscopy was performed on 120 women, from which a group of 96 women with endometriosis and 24 women with other diagnoses (symptomatic controls) were derived. An additional 18 healthy women who were not undergoing surgery were recruited as asymptomatic controls. After the exclusion of women with adenomyosis (n = 3), controls on Lupron (n = 3), the removal of incomplete samples (n = 2), nondetects (n = 1), and amalgamation of control groups, the final study population was 129 women: 93 cases and 36 controls. Of the 93 cases, 68 women were not receiving treatment for endometriosis or were only managing their pain symptoms with NSAIDs or narcotic analgesics, while 25 were receiving treatment for endometriosis including oral contraceptives and Lupron. The putative biomarkers of endometriosis were statistically compared between the symptomatic and asymptomatic controls and did not differ. Thus, the control groups were combined (n = 36) for all subsequent analyses.

Wessels. BDNF as a clinical marker of endometriosis. Fertil Steril 2016.

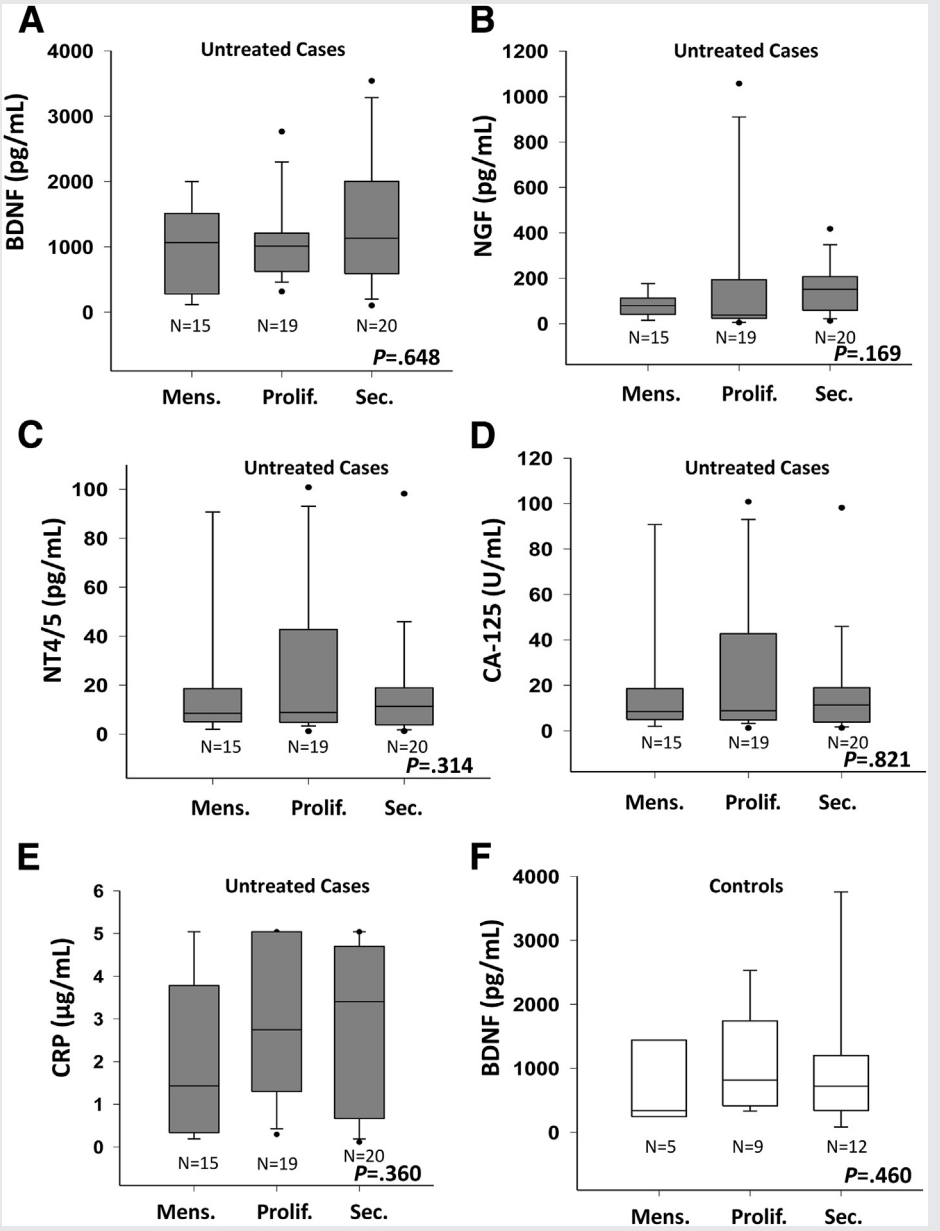
SUPPLEMENTAL FIGURE 2



Amalgamation of asymptomatic and symptomatic control groups. Asymptomatic women who were (n = 6) and were not (n = 12) on oral contraceptives did not have a statistically significant difference (*P*=.174) in circulating BDNF (**A**). Thus, they were combined to increase the sample size of the control group. Symptomatic controls who were (n = 2) and were not (n = 16) on oral contraceptives were also combined because there was no significant difference in circulating BDNF (*P*=.663) (**B**). Circulating concentrations of BDNF (**C**), CA-125 (**D**), and CRP (**E**) were compared between the symptomatic and asymptomatic control groups and did not differ significantly (*P*=.159, .950, and .137, respectively). The two control groups (symptomatic and asymptomatic) were combined into one control group for all subsequent analyses. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data.

Wessels. BDNF as a clinical marker of endometriosis. Fertil Steril 2016.

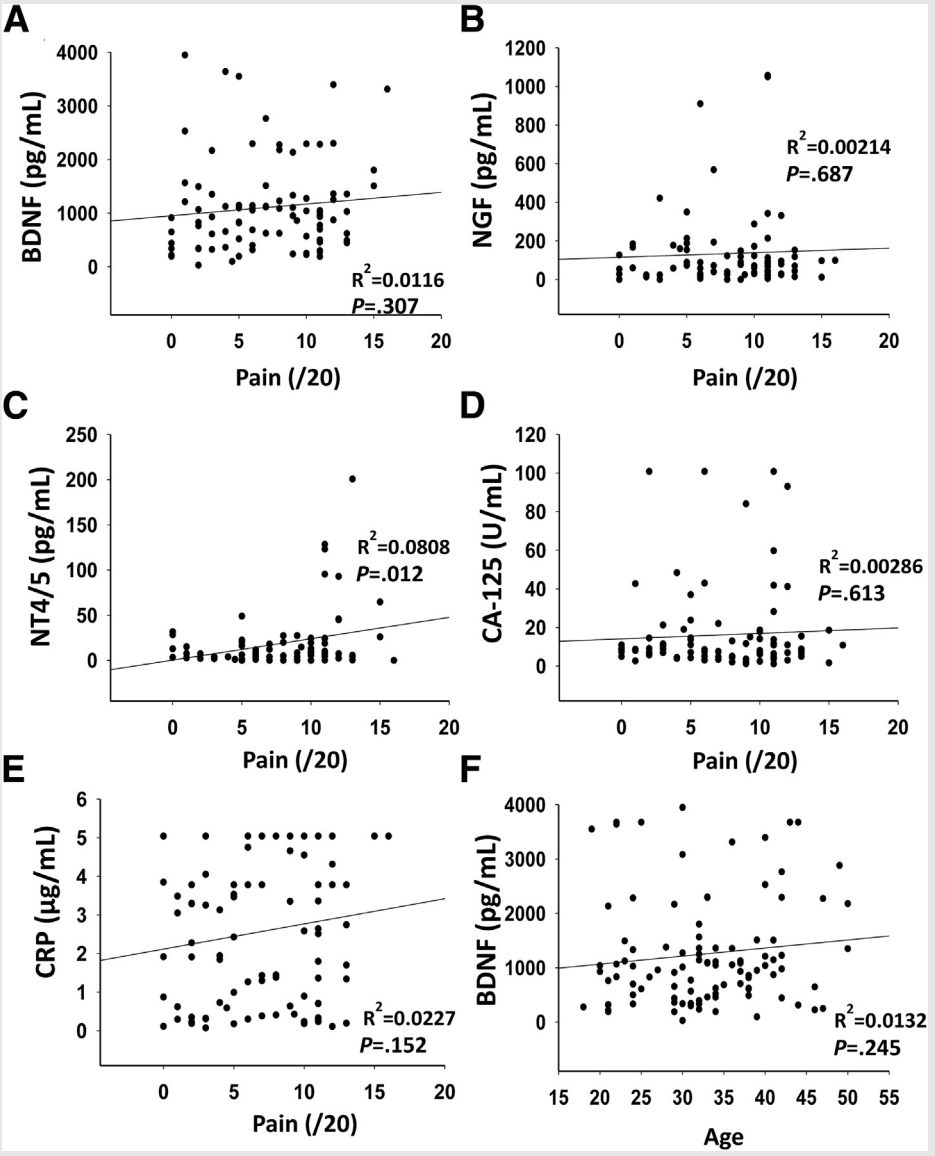
SUPPLEMENTAL FIGURE 3



Effect of menstrual cycle phase on putative clinical markers. The effect of menstrual cycle phase on the concentration of circulating biomarkers in untreated cases (**A–E**) and controls (**F**) was assessed. There were no significant differences in BDNF (P=.648) (**A**), NGF (P=.169) (**B**), NT4/5 (P=.314) (**C**), CA-125 (P=.821) (**D**), or CRP (P=.360) (**E**) across the cycle phases in our study sample of women. Nor was there a significant difference in circulating BDNF across menstrual cycle phase in the controls (P=.460) (**F**). Thus subsequent analyses were not stratified by cycle stage. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles, respectively.

Wessels. BDNF as a clinical marker of endometriosis. Fertil Steril 2016.

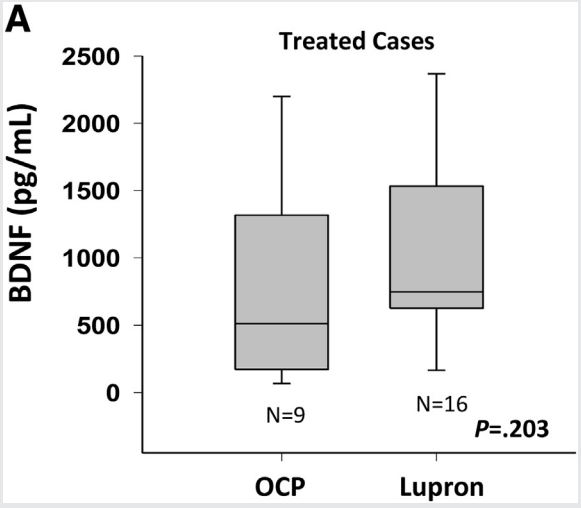
SUPPLEMENTAL FIGURE 4



Effect of pelvic pain and age on putative clinical markers. The relationship between pelvic pain and each putative biomarker was determined by linear regression using pain as the dependent variable in untreated cases and controls. No significant association was observed for BDNF (n = 92; *P*=.307) (**A**), NGF (n = 78; *P*=.687) (**B**), CA-125 (n = 92; *P*=.613) (**D**), or CRP (n = 92; *P*=.152) (**E**). There was a significant relationship between circulating NT4/5 and pain (n = 78; *P*=.012) (**C**). As the majority of markers did not have an association with pain, subsequent analyses were not stratified by pelvic pain. There was no association between circulating BDNF and age (n = 104; *P*=.245) (**F**).

Wessels. BDNF as a clinical marker of endometriosis. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 5



Effect of oral contraceptives and Lupron on circulating BDNF. In the group of 25 women being treated for endometriosis, nine women were on oral contraceptives (OCP) and 16 were on Lupron (16 of 25). There was no significant difference (P=.203) in the concentration of BDNF between women on OCP and those on Lupron (**A**), thus they were grouped together and called the "treated" group in all subsequent analyses. Whiskers on the box plots represent the 10th and 90th percentiles, while the lower limit of the box is the 25th percentile and the upper limit is the 75th percentile. The line within the box is the median of the data.

Wessels. BDNF as a clinical marker of endometriosis. Fertil Steril 2016.